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Conditional Mutation of *SMC5* in Mouse Embryonic Fibroblasts

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Abstract

The structural maintenance of chromosomes (SMC) complex, SMC5/6, is important for genome maintenance in all model eukaryotes. To date, the most extensive studies have focused on the roles of Smc5/6 in lower eukaryotes, such as yeast and fly. In the handful of studies that have used mammalian cells, siRNA was used by most to knockdown SMC5/6 components. RNAi methods have been very important for scientific progression, but they are hindered by incomplete silencing of protein expression and off-target effects. This chapter outlines the use of a conditional knockout approach in mouse embryonic fibroblasts to study the function of the SMC5/6 complex. These cell lines provide an alternative method to study the function and properties of the SMC5/6 complex in mammals.

Keywords

Structural maintenance of chromosomes; SMC5/6; Mouse embryonic fibroblast; Conditional knockout; DNA damage; Chromosome segregation; Micronuclei; DNA bridges; Mitotic catastrophe

1 Introduction

The structural maintenance of chromosomes (SMC) proteins form a highly conserved group of complexes that play a key role in maintaining genomic integrity. SMC proteins are involved in essential chromosome-based processes, such as sister chromatid cohesion, chromosome condensation, and chromosome segregation. Additionally, SMC proteins have been found to play roles in DNA replication, DNA damage repair and transcription [1-6].

The SMC proteins interact with one another to form a V-like structure. In eukaryotes, there are six SMC members that, with other protein components, form three protein complexes: cohesin (SMC1/3), condensin (SMC2/4), and SMC5/6. Cohesin and condensin are known to be involved in two major cell division events: cohesion of sister chromatids, and premitotic chromosome compaction, respectively. Mutations in either of these complexes results in dramatic disruption of chromosome segregation and causes genomic instability [1, 2, 4, 5, 7].

Unlike cohesin and condensin, the role of SMC5/6 is less well-defined. Though mechanistically unclear, studies in budding and fission yeast have shown SMC5/6 to be involved in double-strand DNA break (DSB) repair via homologous recombination (HR) [8], maintenance of replication fork upon stalling [9], and chromosome segregation [10].

To date, most studies that have used mammalian cells to examine the function of SMC5/6 have used siRNA to knockdown SMC5/6 components. However, RNAi methods have been hindered by incomplete silencing of protein expression and off-target effects [11]. This chapter outlines the use of a conditional knockout approach in mouse embryonic fibroblasts to study the function of the SMC5/6 complex. This method involves the use of Cre recombinase to mediate deletion of the essential fourth exon of *Smc5*, which is flanked (floxed) by two *loxP* sites [12]. The Cre recombinase used in this system is tamoxifen-inducible, allowing the investigator to grow cells prior to tamoxifen treatment, and control the timing of *Smc5* deletion. This method allows for specific mutation of the *Smc5* gene, subsequent protein depletion, and destabilization of the SMC5/6 complex. Furthermore, we show that conditional knockout of *Smc5* sensitizes cells to hydroxyurea and etoposide exposure, underlining the importance of the SMC5/6 complex in DNA damage response and genome maintenance.

2 Materials

2.1 Cell Lines

1. Previously created heterozygous $Smc5^{del}$ mice [13, 14] are bred to mice harboring the conditional $Cre\text{-}ER^{T2}$ transgene (B6.129-$Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj}$/J, JAX), which resulted in progeny heterozygous for the $Smc5^{del}$ allele and hemizygous for the $Cre\text{-}ER^{T2}$ genotype. These mice are bred to homozygous $Smc5^{flox}$ mice to derive *Smc5* conditional knockout ($Smc5^{flox/del}$, $Cre\text{-}ER^{T2}$) and control ($Smc5^{wt/flox}$, $Cre\text{-}ER^{T2}$) genotypes. In addition, $Smc5^{flox/del}$ mice without the $Cre\text{-}ER^{T2}$ transgene are used to establish an additional control line. The $Cre\text{-}ER^{T2}$ transgene is a fusion protein between Cre recombinase and estrogen receptor T2. ER^{T2} retains the Cre recombinase in the cytoplasm until tamoxifen administration releases this inhibition, thus permitting the recombination of genomic *loxP* sites. A schematic of the $Smc5^{del}$ and $Smc5^{flox}$ alleles and tamoxifen-induced Cre recombination is shown in Fig. 1a. (*See* Note 1 for Mouse Genome Informatics identification numbers).
2. Isolated 13.5 dpc fetuses with these genotypes are used to establish mouse embryonic fibroblast cell lines (MEFs). MEFs are immortalized according to the NIH-3T3 protocol [15]. Primary mouse cells are passaged every 3 days until cells entered senescence. MEFs were monitored for regrowth and passaged until cells resumed a stable growth pattern (passage ~10–15). Cells are stored in liquid nitrogen in freezing medium (20% fetal bovine serum (FBS), 10% DMSO, and 70% cell culture medium) (*see* Subheading 2.2 and Note 2).

1. The Mouse Genome Informatics (MGI) identification numbers for the $Smc5^{flox}$ and $Smc5^{del}$ are MGI:5882565 and MGI:5882566, respectively.
2. All cell lines are available upon request.

2.2 Culture Medium and Solutions

1. Culture medium: DMEM (Corning, Cat. No. 10-013-CV) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (ThermoFisher Scientific, Cat. No. 15140122).
2. Low serum culture medium: DMEM supplemented with 1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin.
3. 1× phosphate-buffered saline (PBS), pH 7.4.
4. 10× 0.5% trypsin–EDTA (no Phenol Red).
5. 1 mM tamoxifen: stock solution of 1 mM (Z)-4-hydroxytamoxifen prepared in absolute ethanol (*see* Note 3).
6. 1 mg/mL nocodazole: stock solution of 1 mg/mL nocodazole prepared in DMSO (*see* Note 3).
7. 100 mM hydroxyurea: stock solution of 100 mM hydroxyurea (HU) prepared in sterile water (*see* Note 3).
8. 50 mM etoposide: stock solution of 50 mM etoposide prepared in DMSO (*see* Note 3).

2.3 DNA Analysis

1. DNA purification: GeneJet genomic DNA purification. DNA concentration can be determined using a NanoDrop 2000C or equivalent.
2. Polymerase chain reaction: AccuStart II PCR SuperMix (QuantaBio) and 0.1 μM of each primer. Standard thermal cycler with heated lid can be used (e.g., Bio-Rad T100 Thermal Cycler).
   - Primer #1 (5′-ACTCAGTCTCACACGGCAAG-3′)
   - Primer #2 (5′-ATCCTTCCCACCTTGGAAAC-3′)
   - Primer #3 (5’-GAGATGGCGCAACGCAATTAAT-3′)
   - Primer #4 (5′-AGAAAGACATCAAACTAACCGCTGGC-3′)
     - Expected product sizes are listed in Fig. 1a
3. 1% Cresol red: Suspend 17 g of sucrose with molecular grade water, make up to 49 mL. Add 1% w/v cresol red dye.
4. Agarose gel, buffer and equipment: 1.5% agarose suspended in 1 × TBE buffer (10.8 g Tris base, 5.5 g boric acid, 4 mL 0.5 M Na2EDTA (pH 8.0), make up to 1 L in milli-Q water). Standard agarose gel electrophoresis equipment can be used.

3.Tamoxifen, nocodazole, hydroxyurea, and etoposide are hazardous mutagenic/carcinogenic chemicals. Follow the safety precautions and guidelines for these chemicals.

2.4 Protein Analysis

1. Protein extraction buffer: RIPA lysis buffer system (Santa Cruz) with 1× protease inhibitor (Roche) and 1× PhosSTOP phosphatase inhibitor (Roche).
2. Bioruptor Plus sonication system (Diagenode) or equivalent.
3. Pierce BCA protein assay kit (ThermoFisher Scientific). Protein concentration readings can be obtained using a NanoDrop 2000C or equivalent.
4. Loading buffer: 4× Laemmli sample buffer with 10% v/v 2-mercaptoethanol.
5. 4–15% gradient SDS-PAGE gel.
6. 10× SDS PAGE running buffer: 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS make up to 1 L with milli-Q water.
7. Mini-PROTEAN Tetra Cell or equivalent.
8. PVDF membranes.
9. TransBlot turbo system or equivalent.
10. Phosphate-Buffered Saline (PBS): 10× PBS solution pH 7.4 diluted to 1× PBS using in milli-Q water.
11. 3% BSA diluted in 1× PBS.
12. Clarity western ECL substrate (Bio-Rad) or equivalent.
13. Primary antibodies:
    - 1:400 dilution rabbit anti-SMC5 (Bethyl Laboratories Inc),
    - 1:400 dilution rabbit anti-SMC6 (Abcam)
    - 1:200 dilution rabbit anti-p53 (Santa Cruz)
    - 1:1000 dilution rabbit anti-acetyl-p53 (Lys379) (Cell Signaling)
    - 1: 20,000 dilution mouse anti-α-tubulin (Sigma)
14. Secondary horseradish peroxidase (HRP) conjugated antibodies:
    - 1:4000 dilution goat anti-rabbit, HRP (ThermoFisher Scientific)
    - 1:4000 dilution rabbit anti-mouse, HRP (ThermoFisher Scientific)

2.5 Microscopy

1. 75 × 25 mm premium frosted microscope slides.
2. 12 mm diameter circular cover glass.
3. 0.1% gelatin diluted in distilled water.
4. 10% formalin solution neutral buffered.
5. 0.1% Triton X-100.

6. 1× TBST: 10× Tris buffered saline diluted to 1× using milli-Q water, add 0.1% Tween 20.
7. Antibody blocking reagent: 4% horse serum diluted in 1× PBS.
8. Primary antibodies:
   - 1:100 dilution rabbit anti-Rad51 (ThermoFisher Scientific)
   - 1:100 dilution human anti-centromere (Antibodies Inc)
   - 1: 1000 dilution mouse anti-α-tubulin (Sigma)
9. Secondary Alexa Fluor (AF) conjugated antibodies:
   - 1:3000 dilution goat anti-rabbit, AF568 (ThermoFisher Scientific).
   - 1:3000 dilution goat anti-human, AF568 (ThermoFisher Scientific).
   - 1:3000 dilution goat anti-mouse, AF488 (ThermoFisher Scientific).
10. Mounting medium: Vectashield with DAPI (Vector Labs).
11. Microscope: Zeiss CellObserver Z1 microscope linked to an ORCA-Flash 4.0 CMOS camera (Hamamatsu).
12. Zeiss Immersol 518F.
13. Zeiss ZEN 2012 blue edition image software.

3 Methods

3.1 *Growth of Mouse Embryonic Fibroblasts and Conditional Mutation of* Smc5

1. Take a frozen vial of experimental (*Smc5*$^{flox/del}$, *Cre-ER*^{T2} and control (*Smc5*$^{wt/flox}$, *Cre-ER*^{T2} genotypes from liquid nitrogen stocks. Thaw cells and plate into cell culture medium. Monitor cell growth. Normally it takes 3–5 days for these MEFs to achieve 80–90% confluency. Once subconfluent cultures are obtained, MEFs should be passaged every 3 days.
2. To passage, wash cells twice with 1× PBS, and then treat with 0.05% trypsin-EDTA for 2–3 min for detachment. After neutralizing trypsin-EDTA with cell culture medium, cells should be counted using a hemocytometer and plated at a density of 10,000 cells/cm^2.
3. Induce Cre-mediated deletion of the *Smc5*^{flox} allele by treating cells with 0.2 μM (Z)-4-hydroxytamoxifen (4-OH TAM). The 4-OH TAM in cell culture medium should be replenished every 2 days (*see* Note 4). Collect MEFs after 3, 6, and 9 days of 4-OH TAM treatment. Alternatively, collect 5 and 10 days after 4-OH TAM treatment. Parallel cultures without the addition of 4-OH TAM should be used as controls for each time point. Cell proliferation should be monitored and

[4]It is important to maintain 4-OH TAM in culture medium throughout the experiments to ensure that all cells are targeted for *Smc5* deletion. If tamoxifen is removed during an experiment there is the possibility that some cells may still carry a *Smc5*^{flox} allele. *Smc5* deletion results in slowed growth and genome instability. Therefore, a population of cells that still carry the *Smc5*^{flox} allele would outgrow the successfully mutated population, skewing the results.

compared by counting MEFs via a hemocytometer at each time point. MEFs collected should be spun down at 200 × *g* for 3–5 min, washed in 1× PBS. Between 0.1 and 0.5 × 10^6 cells should be collected for DNA analysis, the remaining cells should be spun down, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis.

3.2 *Analysis of Conditional Mutation of* Smc5

1. Process 0.1–0.5 × 10^6 cells collected for DNA extraction using the genomic DNA purification kit, following the user manual provided.
2. Use 50 ng of DNA for each cell sample for PCR Set up 50 μL using the AccuStart II PCR SuperMix user manual provided, and primers listed in **item 2** of Subheading 2.3.
3. PCR conditions:
   - Step 1: 94 °C for 1 min.
   - Step 2: 94 °C for 20 s.
   - Step 3: 58 °C for 30 s.
   - Step 4: 72 °C for 1 min.
   - Repeat **steps 2–4** for 30 cycles.
   - Step 5: 72 °C for 10 min.
4. Run PCR samples on 1.5% agarose gel at 120 V in 1× TBE buffer, using standard agarose gel electrophoresis equipment. An example of the results obtained in experimental and control cell lines are presented in Fig. 1b.

3.3 *Analysis of Protein Levels in Response to Conditional Mutation of* Smc5

1. Extract protein from cell samples by lysing 20,000 cell/μL in protein extraction buffer (**item 1**, Subheading 2.4) on ice.
2. To enhance cell lysis, sonicate at high intensity for 5 min with 30 s on/off intervals, and centrifuged at 10,000 × *g* for 10 min. Transfer the supernatant into a fresh 1.5 mL tube discard the pellet.
3. Measure protein concentration using BCA assay kit, following the user manual provided.
4. Prepare lysates containing 30 μg of protein for each time point and mix with loading buffer. Heat samples at 95 °C for 5 min on heating block.
5. Load proteins extracts on a 4–15% gradient SDS PAGE gel. Run SDS PAGE gel at 100 V in 1× SDS PAGE running buffer until dye front reaches the bottom of the gel.
6. Transfer proteins from gel onto PVDF membrane using the standard program (up to 1.0 A; 25 V constant for 30 min) of the TransBlot turbo system or equivalent.

7. Block membrane in 3% BSA in PBS for 1 h at room temperature. Rinse membrane twice in PBS.
8. Incubate membrane in primary antibody diluted in 3% BSA in 1× PBS for 1 h at room temperature (**item 8**, Subheading 2.5).
9. Wash membrane five times for five minutes with 1× PBS + 0.05% Tween.
10. Incubate membrane in secondary antibody diluted in 3% BSA in 1× PBS for between 30 min and 1 h at room temperature (**item 8**, Subheading 2.3).
11. Wash membrane five times with 1× PBS + 0.05% Tween and once with 1× PBS.
12. Wet membrane with Clarity western ECL substrate following the user manual provided.
13. Capture western blot ECL signal using a Syngene G:Box XR5 or similar. Examples of the results obtained in experimental and control cell lines are presented in Fig. 1c, d.

3.4 *Exposure of MEFs to Hydroxyurea Following Conditional Mutation of* Smc5

1. Plate MEFs at a density of 10,000 cells/cm^2 in cell culture medium and grow with and without 0.2 μM 4-OH TAM for the duration of the experiment.
2. On day 3, passage cells at a density of 10,000 cells/cm^2 in cell culture medium.
3. On day 4, wash once in 1× PBS and switch cells to low-serum medium for 48 h to enrich cells at G1 phase.
4. Wash cells once in 1× PBS. Then add regular cell culture medium containing 2 mM hydroxyurea for 24 h.
5. Wash cells twice with 1× PBS, and then treat with 0.05% trypsin-EDTA for 2–3 min for detachment. After neutralizing trypsin–EDTA with cell culture medium, plate MEFs 20,000–30,000 cells onto a sterile glass coverslip coated with 0.1% gelatin. Culture in regular cell culture media for 24 h.
6. Fix cells in 10% formalin for 20 min at room temperature.
7. Wash coverslips twice with 1× PBS and store at 4 °C for immunostaining (go to Subheading 3.6).

3.5 *Exposure of MEFs to Etoposide Following Conditional Mutation of* Smc5

1. Plate MEFs at a density of 10,000 cells/cm^2 in cell culture medium and grow with and without 0.2 μM 4-OH TAM for the duration of the experiment.
2. On day 3, passage cells at a density of 10,000 cells/cm^2 in cell culture medium.
3. On day 4, add 0.1 μg/mL of nocodazole for 24 h to enrich cells at G2 phase.
4. Wash cells twice with 1× PBS, and then treat with 0.05% trypsin-EDTA for 2–3 min for detachment. After neutralizing trypsin–EDTA with cell culture medium,

plate MEFs 20,000–30,000 cells onto a sterile glass coverslip coated with 0.1% gelatin. Culture in regular cell culture media for 2 h.

5. Supplement media with 15 mM etoposide for 12 h.
6. Wash cells once in 1× PBS.
7. Culture in regular cell culture media for 12 h.
8. Fix cells in 10% formalin for 20 min at room temperature.
9. Wash coverslips twice with 1× PBS and store at 4 °C for immunostaining (go to Subheading 3.6).

3.6 Preparation of Microscopy Slides for Analysis

1. Permeabilize cells on coverslip with 0.1% Triton X-100 for 10 min at room temperature.
2. Wash once in TBST and block with 4% horse serum in 1× PBS.
3. Incubate coverslips with antibody blocking buffer for 30 min at room temperature.
4. Incubate coverslips with primary antibodies for 1 h at room temperature (**item 8**, Subheading 2.5).
5. Wash coverslips in TBST three times for 5 min.
6. Incubate coverslips with secondary antibodies for 1 h at room temperature in the dark (**item 8**, Subheading 2.5).
7. Wash coverslips in TBST three times for 5 min.
8. Mount coverslips onto microscope slides using Vectashield + DAPI.

3.7 Microscopy and Image Analysis

1. Turn on computer, light source, and microscope.
2. Open ZEN software.
3. Add a drop of immersion oil to the slide and place on top of the 40× objective lens.
4. Focus cells using the filter for DAPI.
5. Locate an ideal field of view that has evenly distributed cells that are in focus within a region of interest that is 512 × 512 pixels.
6. Set optimal exposure time for each channel being imaged. Keep exposure times the same for each image captured.
7. Set optimal Z-stack range and intervals to capture all in-focus light within the region of interest.
8. Click start experiment to capture the Z-stack image.

9. Use the Zeiss Nearest Neighbor 3D Deconvolution module to reduce image noise and relocate stray light to its origin of the Z-stack image.
10. Use the Zeiss Extended Depth of Focus wavelet algorithm module to extract the sharp details at different focus positions from the acquired Z-stacks and combine them to create a single optimal 2D image.
11. Save the final image as a .czi file, and export single channel and merged images as .TIFF or .JPEG files without compression.
12. Image files can then be analyzed and counted for the presence of cells with micronuclei (Fig. 2a, b) or undergoing aberrancies during mitosis (Fig. 2a–c, f).
13. RAD51 foci can be scored manually or using a counting script such as the FindFoci plugins on ImageJ (Fig. 2d, e) [16].

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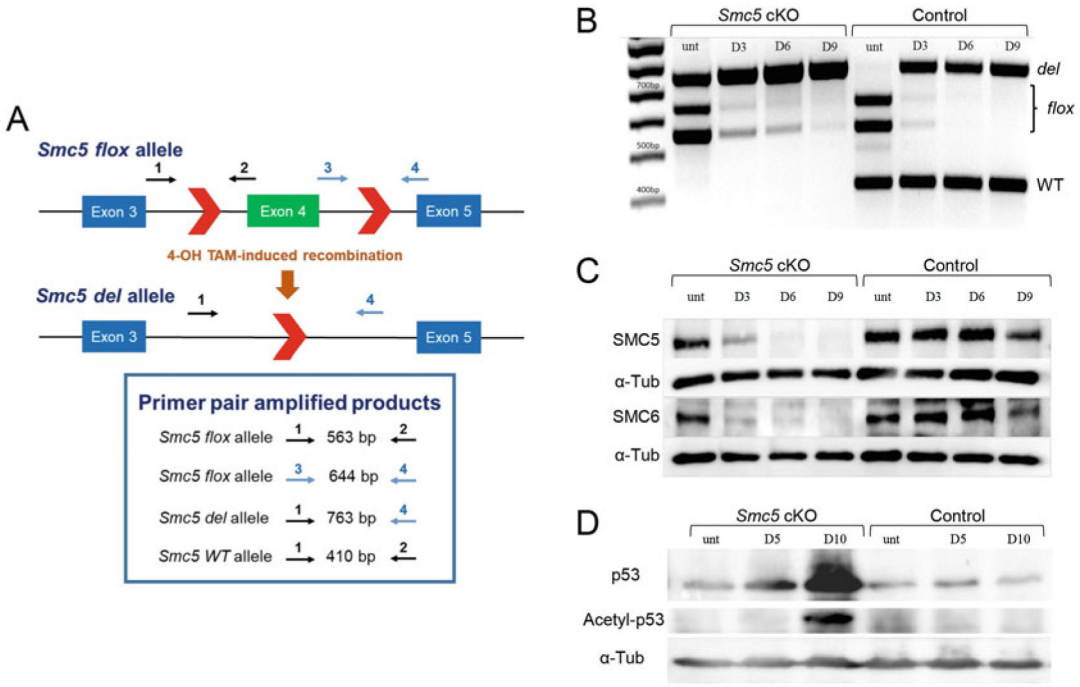

Fig. 1.

Tamoxifen-induced conditional knockout of *Smc5* in mouse embryonic fibroblasts. (**a**) Schematic of $Smc5^{flox}$ and $Smc5^{del}$ alleles. The black and blue arrows numbered 1–4 represent primers used to assess genotype. Exon 4 is flanked by *loxP* sites (red block arrows). Upon 4-OH TAM treatment the $Smc5^{flox}$ allele undergoes Cre-mediated recombination to delete exon 4, which is critical for functional gene expression. The lower panel describes the PCR product sizes expected for the $Smc5^{flox}$, $Smc5^{del}$, and *Smc5* wild-type alleles. (**b**) PCR genotyping of *Smc5* conditional knockout (cKO; $Smc5^{flox/del}$, $Cre\text{-}ER^{T2}$) and control ($Smc5^{wt/flox}$, $Cre\text{-}ER^{T2}$) MEFs after 3, 6, and 9 days (D3, D6, and D9) of treatment with 4-OH TAM (unt = untreated). (**c**) Western blot analysis of SMC5 and SMC6 protein levels during treatment with 4-OH TAM. After 3 days of 4-OH TAM treatment, the protein levels of SMC5 and SMC6 drop to ~10% of levels observed prior to treatment and are almost undetectable by 6 days of treatment. This demonstrates that the stability of SMC6 is dependent on the presence of SMC5. (**d**) Western blot analysis of p53 protein and acetyl-p53 (Lys379) levels during treatment with 4-OH TAM. The *Smc5* cKO MEFs show high levels of p53 and Acetyl-p53 (Lys379) following 10 days of 4-OH TAM treatment. Mouse p53 becomes acetylated at Lys379 (Lys382 in human) to enhance p53-DNA binding in response to DNA damage [17]. α-Tubulin (α-Tub) is used as a loading control for (**c**) and (**d**)

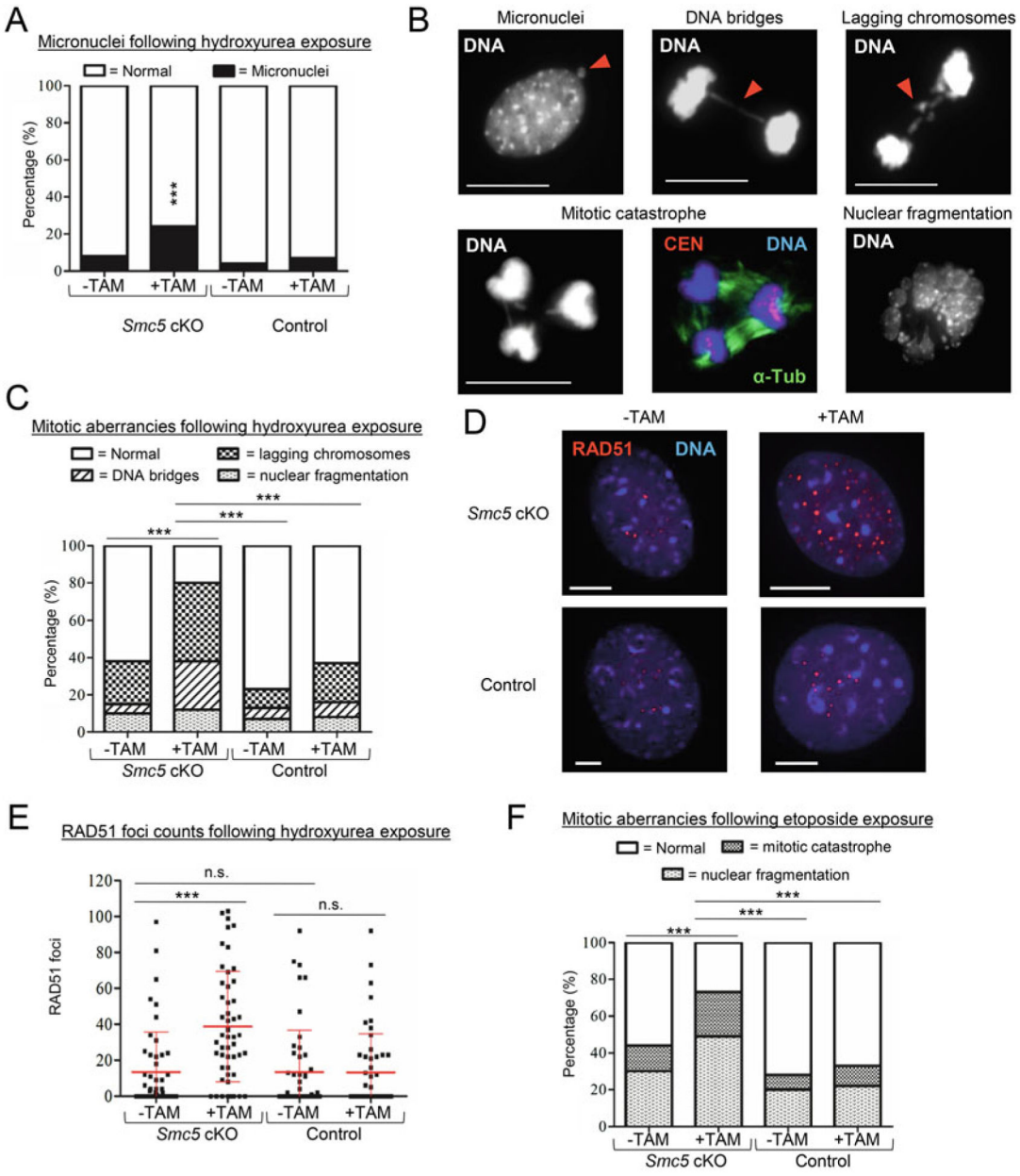

Fig. 2.

Conditional mutation of *Smc5* in MEFs results in hypersensitivity to hydroxyurea and etoposide. (**a**) The *Smc5* cKO MEFs treated with 4-OH TAM and hydroxyurea displayed a significant increase of cells containing micronuclei ($N = 100$ nuclei per condition, repeated three times). Based on Chi-square test; p value <0.0001 (***). (**b**) Examples of DNA morphology observed following hydroxyurea and etoposide treatment. DNA was stained with DAPI, and in one example the cell was also immunolabeled with antibodies for a kinetochore/centromere marker (CEN, red) and alpha-tubulin (α-Tub, green). Scale bar equals 10 μm. (**c**) The *Smc5* cKO MEFs treated with 4-OH TAM and hydroxyurea displayed a significant increase of mitotic cells with lagging chromosomes and DNA bridges ($N = 100$ nuclei per condition, repeated three times). Based on paired, two-tailed t-tests, p values

<0.0001 (***) when comparing DNA bridges and lagging chromosomes observed for *Smc5* cKO in the presence of 4-OH TAM to the other three conditions. (**d**) Example images of RAD51 foci (red) observed following hydroxyurea treatment for *Smc5* cKO and control MEFs in the presence and absence of tamoxifen. DNA was stained with DAPI (blue). (**e**) The *Smc5* cKO MEFs treated with 4-OH TAM and hydroxyurea displayed a significant increase in RAD51 foci counts ($N = 50$ nuclei per condition). Based on Mann–Whitney, two-tailed *t*-test, *p* value <0.0001 (***) when comparing RAD51 foci numbers for *Smc5* cKO in the presence of 4-OH TAM to the other three conditions. Scale bar equals 10μm.(**f**) Following 6 days of culture in the presence or absence of 4-OH TAM (TAM), *Smc5* cKO and control MEFs were treated with etoposide for 12 h ($N = 100$ nuclei per condition, repeated three times). The *Smc5* cKO MEFs treated with 4-OH TAM and etoposide displayed a significant increase of mitotic catastrophe and nuclear fragmentation. Based on paired, two-tailed *t*-tests, *p* values <0.0001 (***) when comparing mitotic catastrophe and nuclear fragmentation observed for *Smc5* cKO in the presence of 4-OH TAM to the other three conditions